NIH275.001NP2

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Jessouroun, Ellen, et al.
App. No	:	10/566,898
Filed	:	October 26, 2006
For	:	PROCESS FOR PREPARING POLYSACCHARIDE-PROTEIN CONJUGATE VACCINES
Examiner	:	Archie, Nina
Art Unit	:	1645
Conf #	:	9576

DECLARATION OF CHE-HUNG ROBERT LEE, PH.D.

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, Che-Hung Robert Lee, declare as follows:

1. I am one of the inventors of the subject application. I have a Ph.D. in Chemistry and 11 years of experience working in research and development on vaccine conjugation. I am currently affiliated with the U.S. Food and Drug Administration. Over my career, which has spanned 35 years, I have published more than 120 papers and abstracts, given numerous invited talks throughout the country, and am an inventor on 6 patents and patent applications.

2. I am familiar with the prosecution of the present application, including the Office Action Made Final mailed May 19, 2008, the references cited therein, and the Amendment After Final accompanying this Declaration.

3. I am advised that pending Claims 1-14 have been rejected as obvious over U.S. Patent No. 5,849,301 ("Lees") in view of U.S. Patent No. 5,773,007 ("Penney et al.") and U.S. Patent No. 6,756,040 ("Peetermans et al.").

Appl. No. : 10/566,898
Filed : October 26, 2006

4. The subject matter of Claims 1-14 is directed to methods for preparing conjugate vaccines by reacting an aldehyde activated polysaccharide with a hydrazine activated protein. As acknowledged in the Office Action, Lees does not disclose a pH of 5 to 7 for the conjugation reaction. Lees also does not disclose buffer exchanging the aldehyde-activated polysaccharide to a pH of from 7 to 8 and buffer exchanging the hydrazine-activated protein to a pH of from 10.5 to 11.0 prior to the conjugation reaction. Likewise, neither Penney et al. nor Peetermans et al. includes teachings as to these particular reaction conditions.

5. There have been no publications or patents describing a method for preparing conjugate vaccines using reductive amination reaction of hydrazide and aldehyde. Likewise, no commercial conjugate vaccine is prepared by reductive amination reaction of hydrazide and aldehyde. It was recognized that methods utilizing protein modification with hydrazide by carbodiimide (EDC) lead to insolubility and precipitation problems in the product. As such, protein modification with hydrazide by EDC was deemed not suitable for use in conjugate vaccine preparation (see, e.g., Schneerson et al, J. Exp. Med. 152:361-376, 1980, page 370, lines 6-7 from bottom; and Chu et al, Infection and Immunity, 40:245-256, 1983, page 246, left column, last paragraph, both submitted in an Information Disclosure Statement dated January 17, 2008). I and my co-inventors have developed a method that does not exhibit the insolubility and precipitation problems of EDC-catalyzed hydrazide-activated protein methods. This is accomplished by maintaining the hydrazide-activated protein soluble at pH 10.5-11. We have successfully applied our methodology to the preparation of conjugate vaccines via a reductive amination reaction with an aldehyde-activated polysaccharide. The covalent linkage created the conjugate vaccines as presently claimed is:

protein-C(=O)-*NH*-*NH*-CH_2-polysaccharide

The italicized letters denote the inserted spacer or linker between the protein and the polysaccharide.

6. In the Examiner's Response to Applicants' Arguments in the Office Action dated May 19, 2008, the Examiner cites to various passages of Lees as teaching applications method:

> Therefore Lees A teaches a method for preparing a conjugate vaccine, the method comprising: reacting a polysaccharide with an oxidizing agent (sodium periodate), whereby a solution of an aldehyde-activated polysaccharide is obtained; reacting a protein with hydrazine at an acidic pH (see column 6, lines 65-67, column 8 lines 1-3, "reaction of hydrazides"), whereby a solution of a

> hydrazine-activated protein is obtained; whereby a conjugate is obtained; and neutralizing unreacted aldehyde groups with acidic [sic] acid dihydrazide, whereby a conjugate vaccine capable of stimulating an immune response is obtained, wherein the oxidizing agent comprises NaIO4 (see column 5 line 27-32), wherein the solution of the aldehyde-activated polysaccharide is buffer exchanged with a HEPES buffer, wherein the solution of the aldehyde-activated polysaccharide is buffer exchanged to a pH of from about 7 to about 8 (column 11 lines 55-65, wherein the solution of the hydrazine-activated protein is buffer exchanged with a carbonate buffer (column 11 lines 55-65), wherein the solution of the hydrazine-activated protein is buffer exchanged to a pH of from about 10.0 to about 11.0, wherein a pH of the solution of the hydrazine-activated protein is raised to from about 7.0 to about 11 before the solution of the hydrazine-activated protein is buffer exchanged to a pH of from about 10.0 to about 11.0, whereby substantially all unreacted compounds and unconjugated polysaccharides are removed, yielding a purified conjugate vaccine ...

7. I disagree with the Examiner's conclusions as to what these particular passages of Lees teach. The method of Lees, unlike my and my co-inventors' method, uses the cyanylating agent 1-cyano-4-dimethylammoniumpyridinium tetrafluoroborate (CDAP) to activate polysaccharide with cyanate groups at pH 9-10 prior to conjugation to a protein or a limited or minimally hydrazide-activated protein (see Lees, column 7, third paragraph). Lees method using CDAP does not require the protein or hydrazide-activated protein to be maintained at pH 10.5-11 in order to avoid aggregation and precipitation. Furthermore, the covalent linkage created in the conjugated vaccines of the Lees method is:

protein-NH-*C(=NH)*-O-polysaccharide

and/or

limited protein-C(=O)-*NH-NH-C(=NH)*-O-polysaccharide

8. The fundamental difference between my and my co-inventors' method as claimed and that of and Lees is that our method uses a reductive amination reaction between hydrazide and aldehyde groups, and Lees method uses a reaction between cyanate and amino or hydrazide groups. Because of this fundamental deficiency of the teachings of Lees and its lack of relevance to our method as claimed, a person of ordinary skill in the art would not arrive at our method as claimed by modifying the teachings of Lees or by combining the teachings of Lees with those of Penney et al. and Peeterman et al.

9. As discussed in the application as filed, conventional methods for the synthesis and manufacture of polysaccharide-protein conjugate vaccines, such as the method disclosed in

Lees, employ conjugation reactions with low efficiencies (typically about 20%). Referring to Lees, Table 3, Example 7 specifies an efficiency of 20.4%. Most efficiencies reported in Lees are much lower (3.1 to 11.3 %). In contrast, the method as presently claimed exhibits a dramatically higher conjugate yield (typically as high as 60%). The improved efficiency is due primarily to maintaining the activated protein in a form with improved solubility prior to conjugation. This is accomplished by maintaining the pH of the activated protein at a pH of from 10.0 to 11.0 by buffer exchanging prior to the conjugation step. The conjugation reaction is then conducted under neutral/mild acidic conditions (pH 5 to 7), which results in enhanced solubility of the conjugate. Accordingly, the aforementioned features of the method as claimed are linked to a surprising and advantageous effect, namely, improved efficiency. The improved efficiency of my and my co-inventors' conjugation method as claimed has other added benefits including, but not limited to, simpler product purification. For example, removal of small molecule by-products can be achieved by a diafiltration step alone, instead of by a combination of a diafiltration step and a chromatographic separation step as in conventional methods.

10. An experiment was conducted to determine conjugation efficiencies using my and my co-inventors' method. Tetanus toxoid (TT, 4.2 mg/mL) was activated with 0.42 M hydrazine or adipic acid dihydrazide in the presence of 20 mM 1-[3-(dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (EDC), 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5 at 20-24°C. After reacting for 4 hours, the pH of the reaction mixture was raised to 7.5-10 with 1 N NaOH to stop the reaction. The reaction mixture was buffer-exchanged with 30 mM NaCl, 3 mM Na_2CO_3, pH about 10.5 at 4°C using a 12-14 KDa dialysis membrane. The protein concentration of the resulting TT-hydrazide sample was determined by Lowry assay (see Pierce Catalog 2003-2004, page 306) using bovine serum albumin as a standard. The hydrazide content was determined by TNBS assay using adipic acid dihydrazide as a standard, as described in Vidal, J. Immunol. Methods 1986; 86:155-156. The degree of activation of TT so prepared was approximately 50 hydrazide groups per TT molecule.

11. Meningococcal group C polysaccharide (Mn C PS) (10 mg/mL) was reacted with 6 mM $NaIO_4$ at 20-24°C for 4 hours. The sample was dialyzed against 10 mM N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES), pH 7.5 at 4°C using a 12-14 KDa dialysis membrane. The concentration of the resulting activated PS was determined by resorcinol assay using N-acetyl neuraminic acid as the standard with a correction factor of Mn C PS/N-

acetyl neuraminic acid = 1.104/1, as described in Monsigny et al., Anal. Biochem. 1988; 175:525-530. The aldehyde content of the activated polysaccharide was determined by bicinchoninic acid (BCA) assay (Pierce Catalog 2003-2004, pages 241 and 305) using glucose as a standard. The degree of activation of the activated Mn C PS prepared by this protocol was approximately one aldehyde group per 80 monomers.

12. An aliquot of hydrazide-containing TT was adjusted to 25 mg/mL by lyophilization and dissolution in water. An aliquot of aldehyde-containing Mn C PS was adjusted to 25 mg/mL by lyophilization and dissolution in 0.2 M HEPES, pH 7.5, 30 mM ethylenediaminetetraacetic acid (EDTA). The activated TT solution was added to an equal volume of the activated Mn C PS and vortexed. The reaction mixture was incubated at 20-24°C for 18 hours. The reaction mixture was treated with $NaBH_4$ (10-fold molar equivalent to initial aldehyde concentration in the activated PS) for 6 hours. The solution was buffer-exchanged with saline, 10 mM HEPES, pH 7.5, 1 mM EDTA using a 12-14 KDa molecular weight cut-off membrane. Total protein was determined by Lowry assay using bovine serum albumin as a standard. Total Mn C PS content was determined by resorcinol assay using N-acetyl neuraminic acid as a standard, as described in Monsigny et al., Anal. Biochem. 1988; 175:525-530.

13. The unconjugated free Mn C PS in the prepared Mn C PS-TT conjugate was determined by the method of C18 particle absorption of protein in the conjugate product followed by comparing the saccharide signal of the supernatant in high performance size exclusion chromatography (HPSEC) to those of the activated Mn C PS of known concentrations. To estimate the yield of the conjugation reaction, the conjugate product was diluted to approximately 1 mg/mL concentration of Mn C PS. 100 µL of this solution was mixed and incubated with 250 µL of activated C18 particles for an hour with gentle agitation. The supernatant was collected after centrifugation, and the C18 gel was washed twice with 100 µL saline. The combined supernatant and wash was adjusted to 333 µL with saline and passed through a 0.2 um membrane microfilter. The filtrate was analyzed with HPSEC together with standard concentrations of activated Mn C PS at 0.033, 0.067 and 0.134 mg/mL, giving the area of the saccharide signals of these samples as 19.4, 4.8, 9.2, and 18.4, respectively. The saccharide concentration of the filtrate was calculated from the standard curve as 0.141 mg/mL, which was 3.3 times volume of the starting sample. Thus the starting sample contained 0.465 mg/mL (0.141 mg/mL x 3.3) free Mn C PS. The total Mn C PS concentration was determined as

1.131 mg/mL by modified resorcinol assay. The yield was estimated to be about 60% (100% x (1-0.465/1.131)).

14. Figure 1 provides estimation of free polysaccharide in a Mn C PS-TT conjugate product prepared by reductive amination conjugation of aldehyde-activated PS and hydrazide-activated protein. Figure 1A provides HPSEC profiles of an Mn C PS-TT conjugate pre (3) and post (1) C18 absorption, and pure TTH (2) monitored at 280 nm, detecting protein. Complete absorption of protein species by C18 from the conjugate product is shown in profile (1). Figure 1B provides HPSEC profiles of the same three injections as in Figure 1A monitored at 206 nm, detecting protein and polysaccharide. The peak at 22.5 minutes in post C18 absorption (1) is from the un-absorbed free polysaccharide in the conjugate product. Figure 1C provides a comparison of HPSEC profile at 206 nm of free PS in conjugate product (1) with those of activated Mn C PS at 0.033 mg/ml (2), 0.067 mg/ml (3), and 0.134 mg/ml (4).

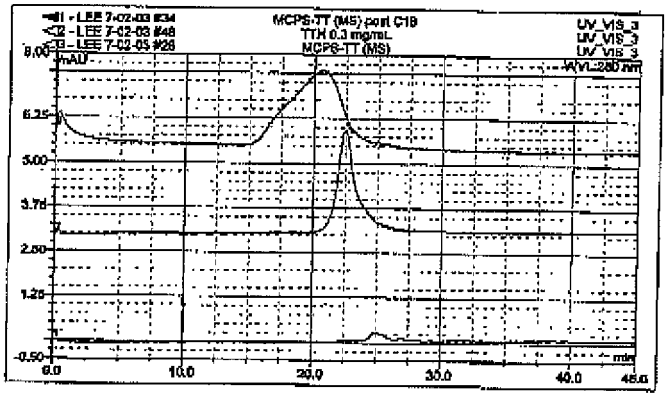

Figure 1A

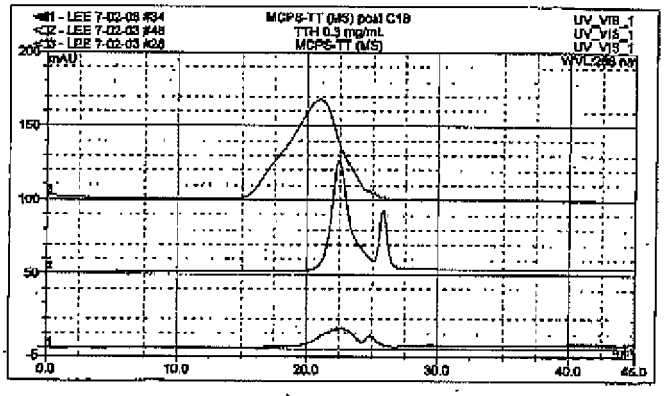

Figure 1B

Appl. No. : 10/566,898
Filed : October 26, 2006

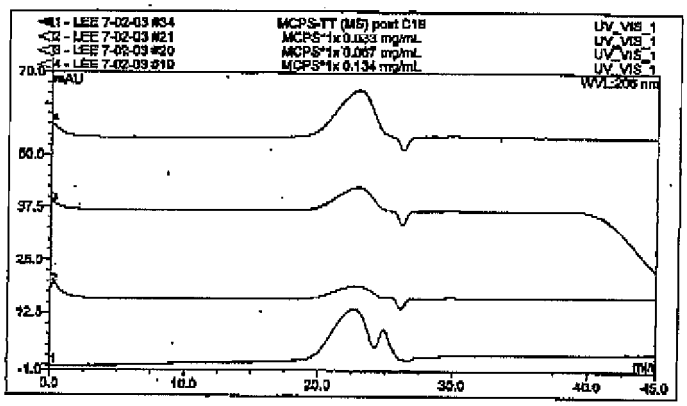

Figure 1C

15. Figure 2 provides a quantitation of free PS in the Mn C PS-TT conjugate prepared by reductive amination conjugation of aldehyde-activated PS and hydrazide-activated protein. The area of the peak at 22.5 minutes in HPSEC profiles 2, 3 and 4 in Figure 1C is measured and plotted against its respective concentration to construct a standard curve. The content of free PS in conjugate product is calculated from the peak area at 22.5 minutes of profile 1 in Figure 1C.

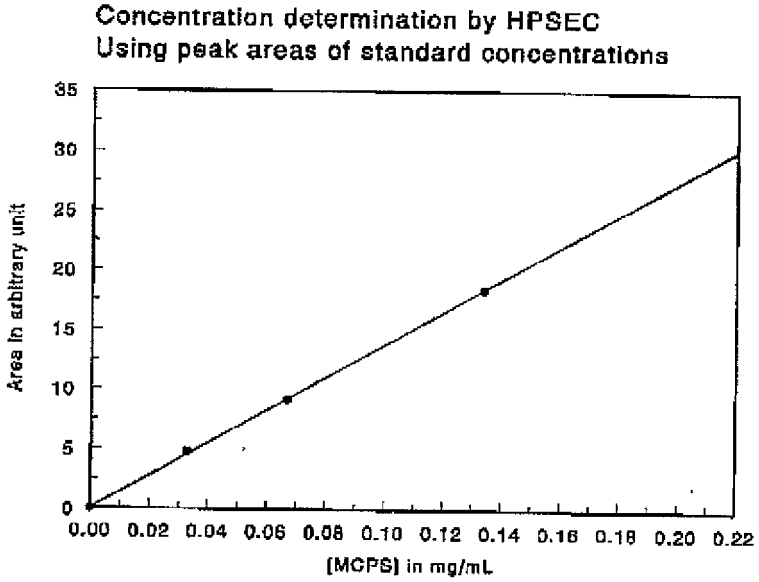

Figure 2

16. I declare that all statements made herein are true, and that all statements made upon information and belief are believed to be true, and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or

Appl. No. : 10/566,898
Filed : October 26, 2006

imprisonment, or both, under 18 U.S.C. § 1001, and that willful, false statements may jeopardize the validity of the application, or any patent issuing thereon.

Dated: 8/20/08 By: Che-Hung Robert Lee
Che-Hung Robert Lee

5677293_3
081508